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Long-Term Administration of D-NAME Induces Hemodynamic and Structural Changes in the Cardiovascular System

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Summary

N^G-nitro-D-arginine-methyl ester (D-NAME) is considered to be an inactive enantiomer of L-NAME and is generally used as the negative control for NO synthase inhibition with L-NAME. With the aim to compare the effects of 4-week L-NAME and D-NAME treatments on hemodynamic and cardiovascular structural parameters, four groups of male Wistar rats were investigated: the controls and groups administered 40 and 20 mg/kg/day of L-NAME and 40 mg/kg/day of D-NAME. At the end of the experiment, myocardial NO synthase activity decreased by 42, 24 and 25 %; aortic NO synthase activity decreased by 35, 15 and 13 % vs. controls in the L-NAME 40, L-NAME 20 and D-NAME 40 groups, respectively. The DNA concentrations in the myocardium and the aorta increased significantly after L-NAME and D-NAME treatments. The inhibition of NO synthase was accompanied by a significant elevation in systolic blood pressure in all three groups. The LVW/BW ratio increased by 27, 14 and 13 % vs. controls in the L-NAME 40, L-NAME 20 and D-NAME 40 groups, respectively. The aortic wall mass, measured as the crossectional area, increased by 45, 17 and 25 % vs. controls in the L-NAME 40, L-NAME 20 and D-NAME 40 groups, respectively. Myocardial fibrosis represented 0.94 % in the controls, but 7.96, 4.70 and 5.25 % in L-NAME 40, L-NAME 20 and D-NAME 40 groups, respectively. It is concluded that D-NAME, although less affective than L-NAME, inhibits NO synthase activity resulting in hemodynamic and structural changes in the cardiovascular system similar to the changes induced by half the dose of L-NAME. Thus, the consideration of D-NAME as an inactive enantiomer and its use as the negative control needs to be reevaluated.

Key words
Nitric oxide synthase • L-NAME • D-NAME • Hypertension • Myocardial fibrosis • Arterial hyperplasia

Introduction

The model of NO-deficient hypertension has gradually found its stable place among traditional models of experimental hypertension. Inhibition of endothelial NO synthase by L-arginine analogues, e.g. N^G-monomethyl-L-arginine (L-NMMA), *N*-iminoethyl-L-ornithine (L-NIO) or N^G-nitro-L-arginine-methyl ester

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(L-NAME) increase blood pressure in rats (Rees *et al.* 1990). NO-deficient hypertension is associated with increased contractility in different parts of the vascular tree (Török *and* Gerová 1996), attenuation of vascular relaxation (Delacretaz *et al.* 1994, Holécyová *et al.* 1996), reduction of heart rate (Pecháňová *and* Bernátová 1996) and decrease of cardiac output (Amrani *et al.* 1992). The structural changes after long-term inhibition of NO synthase in the cardiovascular system include left ventricular hypertrophy (Arnal *et al.* 1993) and extensive areas of fibrosis and necrosis observed after 4 and 8 weeks of L-NAME treatment (Numaguchi *et al.* 1995, Moreno *et al.* 1996, Babál *et al.* 1997). The vascular alterations are characterized by an increased wall-to-lumen ratio in the coronary microvessels and aorta (Numaguchi *et al.* 1995, Babál *et al.* 1997, Bernátová *et al.* 1999), remodeling of the coronary arteries (Kristek and Gerová 1996, Takemoto *et al.* 1997) and smooth muscle cell proliferaton in the arterial media (Babál *et al.* 1997). Aortic smooth muscle cell polyploidy was observed after 3 weeks of L-NAME administration (Devlin *et al.* 1998). The changes in vascular reactivity and medial hyperplasia in coronary arteries are responsible for ischemic injury and development of myocardial fibrosis (Numaguchi *et al.* 1995, Babál *et al.* 1997) and protein remodeling in the heart (Pecháňová *et al.* 1999).

It has been reported that endothelial NO synthase is inhibited by L-arginine analogues in an enantiomer-specific manner (Palmer *et al.* 1988, Chinellato *et al.* 1998). This assumption and the evidence that effects of NO synthase inhibition could be reversed by L-arginine but not by D-arginine, lead to the general adoption of D-stereoisomers of arginine analogues for inactive controls. The majority of these experiments was performed as short-term acute experiments in which no significant effects of D-NAME were evoked (Rees *et al.* 1990, Moreno *et al.* 1997, Chinellato *et al.* 1998). However, D-NAME was reported to increase systolic blood pressure and to decrease the heart rate in some studies (Wang *et al.* 1991, Turner *et al.* 1997) and was also shown to inhibit NO synthase in different areas of the brain (Yamada *et al.* 1995).

The aim of the present study was to examine the potency of long-term treatment with the D-isomer of NAME to induce hemodynamic and structural cardiovascular changes.

Material and Methods

Material

All chemicals were purchased from Sigma Chemie (Diesenhofen, Germany). D-NAME was purchased from both Sigma and Biomol (Plymouth Meeting, USA). The cellulose filter membranes used in the histologic preparations were from Micron Separations, Inc. (Westboro, USA), [^{3}H]-L-arginine was obtained from Amersham International (Little Chalfont, UK).

Experimental model

Male, 15-week-old Wistar rats were randomly divided into 4 groups (n=8 in each group). The first group served as the controls. The second group was given L-NAME 40 mg/kg/day, the third group L-NAME 20 mg/kg/day, and the fourth group was given 40 mg/kg/day D-NAME (n=4: Sigma; n=4: Biomol) in drinking water for 4 weeks. Systolic blood pressure (SBP) was measured daily by the non-invasive method of tail-cuff plethysmography. After 4 weeks, the animals were sacrificed by decapitation. The body weight (BW), heart weight (HW), left ventricle (LVW) and right ventricle weight (RVW) were determined and the LVW/BW as well as RVW/BW ratio were calculated. Apical halves of the ventricles and samples of descending aorta were used for histologic and morphometric analysis. The remaining ventricle and aortic tissues served as samples for NO synthase activity and DNA concentration determination.

Histology

The samples of ventricular tissue were oriented perpendicularly to the sectioning plane. The 3 mm long samples of the aorta (taken 10 mm proximally from the diaphragm) were put in an upright position on cellulose filter membranes to maintain a round shape. The tissues were fixed for 24 h in 10 % phosphate buffered formalin, then routinely processed in paraffin and serial 5 μm thick sections were stained with hematoxylin and eosin and by Van Gieson's staining.

Morphometry

Morphometric evaluation was performed using an Olympus light microscope equipped with a two dimensional image analyzer (Alfa Inotech Corp., USA) as described elsewhere (Babál *et al.* 1997). Van Gieson's staining was applied to enhance the red color contrast of

collagen. Myocardial fibrosis was expressed as percentage of the whole muscle area in three serial sections of each heart ventricles specimen. The area of the aortic wall crossection was measured and changes were expressed in percentage of the area of the controls.

Determination of DNA concentration

Chromosomal DNA concentration in the left ventricle and in the aorta was estimated as previously described (Bernátová *et al.* 1999). Tissue samples (50 mg) were homogenized in 1 ml of 0.5 x SSC (1 x SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at 4 °C. The homogenate was centrifuged at 5000 x*g* for 20 min and the sediment was resuspended in 1 ml of 0.5 x SSC. The same procedure was repeated twice. Then 0.6 ml of SSC and 0.15 ml of 5 % SDS were added to the final sediment, mixed at room temperature for 2 h. NaCl (70 mg) was added to each sample and mixed for 30 min. After centrifugation at 5000 x*g* for 30 min, the supernatant was transferred into polypropylene tubes and 0.75 ml of chloroform-isoamyl alcohol mixture (1:1) was added. The samples were mixed and centrifuged at 5000 x*g* for 30 min. A twofold volume of 96 % ethanol was added to the aqueous phase and kept at –20° C overnight. After centrifugation at 10 000 x*g* for 30 min, the DNA pellet was vacuum-dried, dissolved in sterile water and absorbance at 260 nm measured. DNA concentration was expressed in mg/g of protein.

NO synthase activity

NO synthase activity was determined in crude homogenates of tissue samples by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine as described by Bredt and Snyder (1990), with modifications. Briefly, 50 µl of 10 % homogenate were incubated in the presence of 50 mM Tris-HCl, pH 7.4, 10 µM [^{3}H]-L-arginine (specific activity 5 GBq/mM, approx. 100 000 d/min), 30 nM calmodulin, 1 mM β-NADPH, 3 µM BH_4 and 2 mM Ca^{2+} in a total volume of 100 µl. After 20 min incubation at 37 °C, the reaction was stopped by the addition of 1 ml of ice-cold 20 mM HEPES buffer pH 5.5, containing 2 mM EDTA, 2 mM EGTA and 1 mM L-citrulline. The samples were then applied to 1 ml Dowex 50WX-8 columns (Na^+ form). [^{3}H]-L-citrulline was eluted by 1 ml of water and measured by liquid scintillation counting. NO synthase activity was expressed as pmol L-citruline/min/mg of protein.

Statistical analysis

Data were expressed as mean ± SEM. One way ANOVA combined with Newman-Keuls multiple comparison test was used for analysis. Values were considered statistically significant at $p<0.05$.

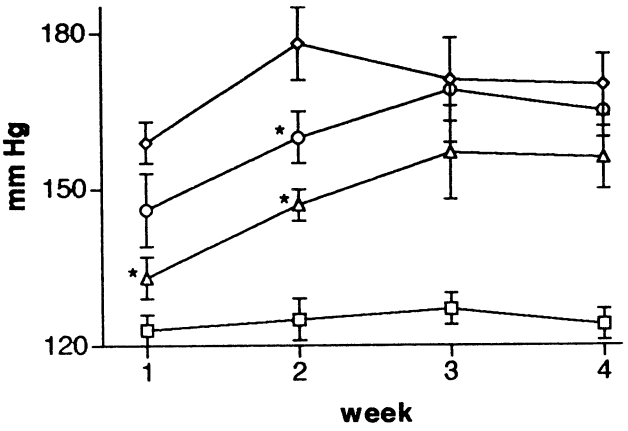

Fig. 1. *Effect of L-NAME and D-NAME administration on systolic arterial pressure. Controls (squares); L-NAME 40 mg/kg/day (rhomboids); L-NAME 20 mg/kg/day (circles); D-NAME 40 mg/kg/day (triangles). All values were significantly higher than controls, * $p<0.05$ vs. L-NAME 40.*

Results

Hemodynamic parameters

After the fourth week of the experiment, SBP was 128±5 mm Hg in the control group. Oral administration of 40 mg or 20 mg/kg/day of L-NAME and 40 mg/kg/day of D-NAME increased SBP by 37, 33, and 27 %, respectively (Fig. 1). At the end of the experiment, LVW/BW ratio was 1.28±0.03 in the control group. This ratio increased significantly in all arginine analogues-administered groups versus the control group (Table 1). The RVW/BW ratio was not affected in any group. No differences between the effects of D-NAME obtained from Sigma and Biomol were noticed and the data were pooled into one group.

Table 1. Effect of 4-week L-NAME and D-NAME treatment on left ventricle weight, myocardial fibrosis and aortic wall mass.

Group (mg/kg/day)	LVW/BW (mg/g)	Myocardial fibrosis (%)	Aortic wall mass (% of control)
Control	1.24 ± 0.03	0.94 ± 0.19	100.00 ± 3.95
L-NAME 40	1.60 ± 0.02***	7.96 ± 0.80***	145.01 ± 4.82***
L-NAME 20	1.46 ± 0.03**$^{+}$	4.70 ± 1.19**$^{+}$	116.92 ± 1.51*$^{+}$
D-NAME 40	1.44 ± 0.03**$^{+}$	5.25 ± 1.03**$^{+}$	125.22 ± 1.36**$^{+}$

****p<0.001, **p<0.01, *p<0.05 vs. control; $^{+}$ p<0.05 vs. L-NAME 40*

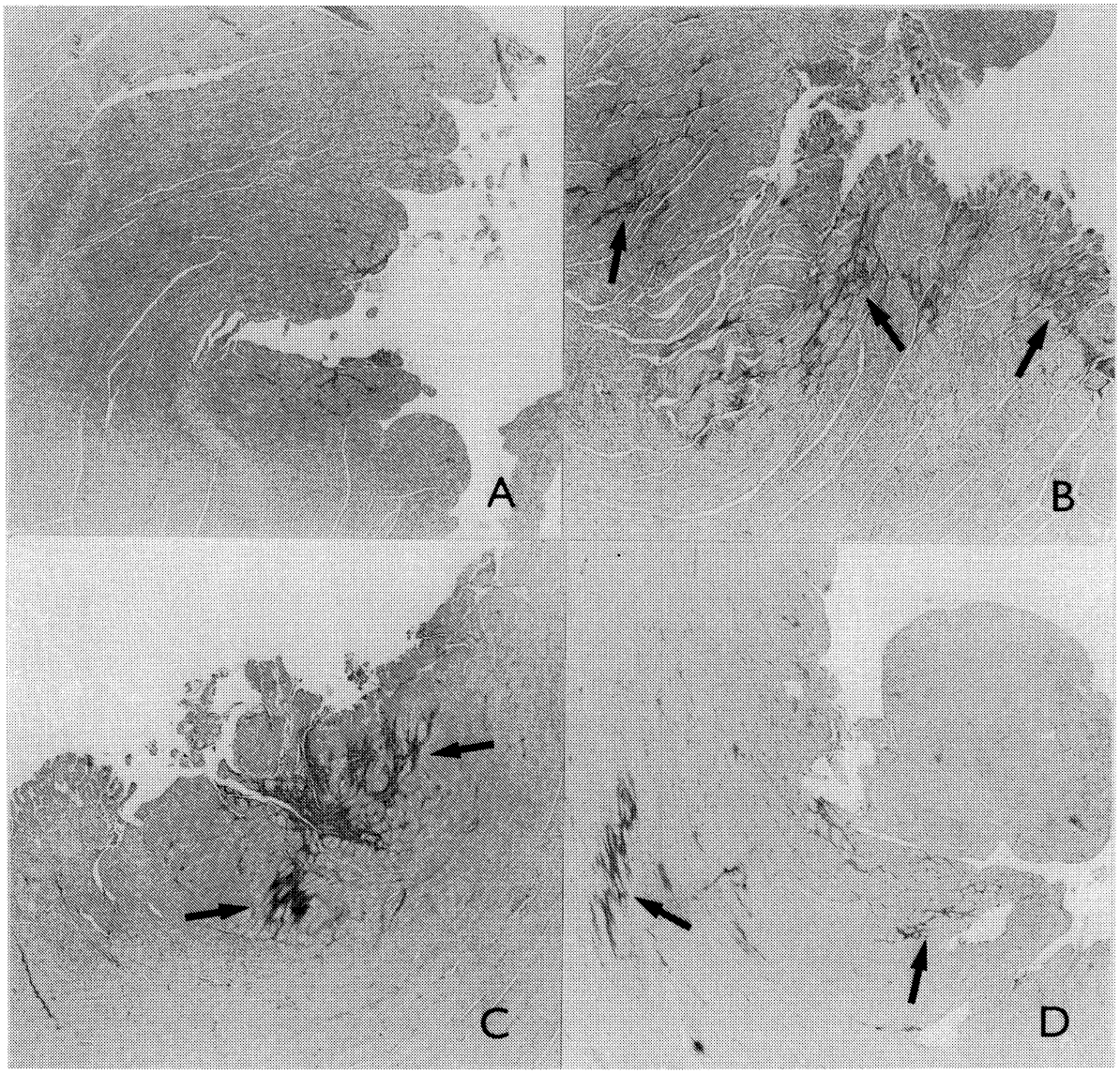

Fig. 2. *Fibrosis development in the left ventricle after 4 weeks of NO-synthase inhibition. Controls* ***[A]****; L-NAME 40 mg/kg/day* ***[B]****; L-NAME 20 mg/kg/day* ***[C]****; D-NAME 40 mg/kg/day* ***[D]****. Fibrotic areas are dark-stained with Van Gieson's staining of collagen (arrow). 140 x.*

Histology with morphometric analysis

Hematoxylin and eosin stained sections revealed large areas of fibrosis in the myocardium of rats administered L-NAME or D-NAME. These changes were accentuated with Van Gieson's staining of collagen (Fig. 2). Fibrotic changes were observed in the left as well as in the right ventricle. Percentual proportion of fibrosis in the heart muscle increased significantly in all experimental groups versus control (Table 1). The magnitude of the crossection area of the aortas documented a significant increase in the wall mass in all groups versus the controls. Differences in the amount of myocardial fibrosis and the aortic wall mass between the 20 mg L-NAME and the 40 mg D-NAME groups were not significant (Table 1).

DNA concentration

Concentration of DNA in the control group was 0.62±0.03 and 1.83±0.18 mg/g protein in the left ventricle and in the aorta, respectively. In all experimental groups there was a significant increase of the DNA concentration versus the controls (Fig. 3).

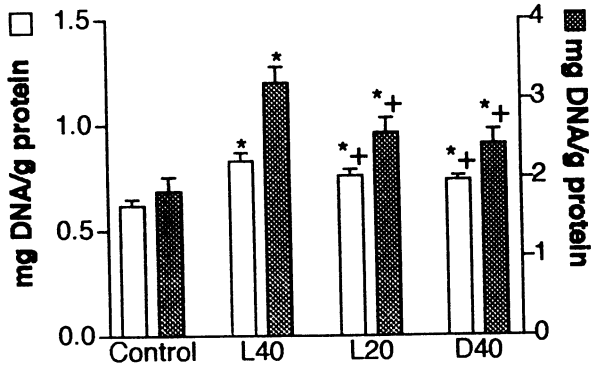

Fig. 3. *Concentration of DNA in myocardium (open columns) and aorta (hatched columns) after 4 weeks administration of L-NAME 40 mg/kg/day (L40), L-NAME 20 mg/kg/day (L20), D-NAME 40 mg/kg/day (D40). * $p<0.05$ vs. controls; + $p<0.05$ vs. L-NAME 40.*

NO synthase activity

In the control group, NO synthase activity was 15.2±0.8 and 20.0±0.7 pmol L-citrulline/min/mg protein in the left ventricle and the aorta, respectively. In the 40 mg L-NAME group, the activity was inhibited by 42 % in the left ventricle and by 35 % in the aorta. In the 20 mg L-NAME and the 40 mg D-NAME groups, NO synthase activity decreased by 24 and 25 % in the myocardium and 15 and 13 % in the aorta, respectively (Fig. 4).

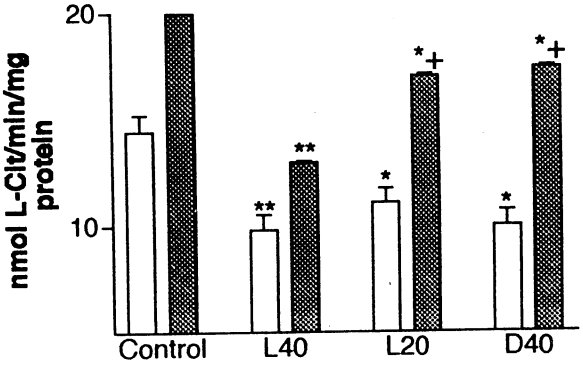

Fig. 4. *NO-synthase activity in myocardium (open columns) and aorta (hatched columns) after 4 weeks administration of L-NAME 40 mg/kg/day (L40), L-NAME 20 mg/kg/day (L20), D-NAME 40 mg/kg/day (D40). ** $p<0.01$, * $p<0.05$ vs. controls; + $p<0.05$ vs. L-NAME 40.*

Discussion

This study has demonstrated that long-term administration of both L-NAME and D-NAME decreases NO synthase activity in the heart and the aorta, and significant elevation of blood pressure in rats. It has been generally accepted that L-arginine analogues, the best known inhibitors of NO synthase, increase blood pressure in acute (Rees *et al.* 1990) as well as in chronic experiments (Gardiner *et al.* 1990, Ribeiro *et al.* 1992). On the other hand, the D-enantiomers of NAME and other analogues of arginine have been used in many studies as inactive controls for NO synthase inhibition with no effect on blood pressure (Rees *et al.* 1990, Moreno *et al.* 1996). These results are contradictory not only to our finding that the blood pressure is significantly increased after D-NAME administration but also to the other reports (Wang *et al.* 1991, Turner *et al.* 1997). Wang *et al.* (1991) described a slower onset of the pressor response to D-NNA, relative to the same dose of L-NNA, in an acute experiment in rats. In the present study, D-NAME increased blood pressure to similar plateau values, but with a significantly slower onset compared to the same dose of L-NAME.

D-NAME induced blood pressure elevation which was accompanied by left ventricular hypertrophy, an increase in DNA concentration and myocardial fibrosis. Our previous findings of increased RNA concentration and [^{14}C]leucine incorporation into proteins of the left ventricle in the long-term L-NAME treated rats (Babál *et al.* 1997, Pecháňová *et al.* 1997) provided direct evidence of increased proteosynthesis typical for the period of developing hypertrophy (Motz *et al.* 1983). The increased DNA concentration in the myocardium after L-NAME and D-NAME treatment may be ascribed to fibrotic tissue enlargement. Proliferating vascular smooth muscle cells could serve as another source for this increase, as is also suggested by a similar DNA concentration increase in the aorta. Apart from the morphological evidence of vascular wall remodeling (Babál *et al.* 1997, Numaguchi *et al.* 1995, Kristek and Gerová 1996), the presence of proliferating smooth muscle cells was confirmed by the demonstration of proliferating cell nuclear antigen-positive cells in the arterial wall after chronic L-NAME treatment (Babál *et al.* 1997)

Our morphometric investigation revealed significant enhancement of fibrotic tissue in the myocardium. Structural changes consisting of extensive areas of fibrosis and necrosis after long-term L-NAME treatment were also observed by others (Numaguchi *et al.* 1995, Moreno *et al.* 1996, Babál *et al.* 1997). This is the first documented evidence that long-term D-NAME treatment, although less effectively than L-NAME, produces structural alterations in the cardiovascular system.

Since D-NAME produced myocardial fibrosis analogously to L-NAME, the critical question was as to whether D-NAME administration also resulted in proliferative vascular changes. Morphometric evaluation of the aorta indicated a significant increase in the vascular wall mass after chronic administration of both enantiomers of NAME.

All the above mentioned hemodynamic and structural changes of the cardiovascular system in the NO-deficient hypertension model are the result of NO synthase inhibition. The decrease of NO synthase activity in several organs after administration of L-NAME has been documented (Yamada *et al.* 1995, Bernátová *et al.* 1996). It appears that D-NAME is capable of exerting the same effect. Determination of NO synthase activity in the left ventricle and in the aorta indicated that D-NAME had approximately one half of the inhibitory potency of L-NAME. Similar results were reported with the NO synthase activity in the brain after 10 days of intraperitoneal administration of L-NAME and D-NAME to rats (Yamada *et al.* 1995). It is noteworthy that the inhibition of the L-arginine/NO pathway by both enantiomers of arginine analogues can be reversed specifically by L-arginine but not D-arginine (Wang *et al.* 1991, Turner *et al.* 1997).

Both L-NMMA and D-NMMA were both reported to moderately attenuate the ischemia/reperfusion-induced changes in lung capillary permeability (Moore *et al.* 1996). This protection of microvascular injury may be related to the ability of L-NAME and D-NAME and NMMA to modify electron transfer through iron centers in the cell, apart from NO synthase (Peterson *et al.* 1992).

We conclude that both L-NAME and D-NAME are efficacious pressor agents in rats. Structural changes in the heart and the aorta induced by chronic administration of 40 mg/kg/day of D-NAME were similar to those elicited by the dose of 20 mg/kg/day of L-NAME. As has also been documented by others (Wang *et al.* 1991, Yamada *et al.* 1995, Turner *et al.* 1997), the D-enantiomers had 30-50 % of the NO synthase inhibitory magnitude of L-NAME and L-NNA. These facts indicate the need for reevaluation of the suitability of D-enantiomers of arginine analogues as "inactive" controls in NO synthase inhibition experiments.

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Abbreviations

L-NAME, N^G-nitro-L-arginine-methyl ester; D-NAME, N^G-nitro-D-arginine-methyl ester; L-NMMA, N^G-monomethyl-L-arginine; L-NIO, *N*-iminoethyl-L-ornithine; L-NNA, N^G-nitro-L-arginine; SBP, systolic blood pressure; LVW, left ventricle weight; RVW, right ventricle weight; BW, body weight.

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